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ORIGINAL ARTICLE

Probiotic potential of bacteriocin-producing *Enterococcus hirae* strain LD3 isolated from dosa batter

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Abstract *Enterococcus hirae* LD3 was isolated from dosa batter and identified by biochemical and molecular techniques such as carbohydrate fermentation pattern and 16S rDNA amplification and sequencing. Strain LD3 demonstrated probiotic properties such as cell surface hydrophobicity, simulated gastric juice, bile salt and acid tolerance, and antibiotic susceptibility. The optimum growth of strain LD3 and bacteriocin production was observed at 37 °C, pH 7.0. Bacteriocin was produced during mid-log phase and found to be thermo-stable, demonstrated activity at an acidic pH range, and was stable in different organic solvents, surfactants, and detergents tested. The antimicrobial activity was not affected by amylase and lipase, but reduced after treatment with pepsin, trypsin, proteinase K, and papain suggesting a proteinaceous nature. Crude bacteriocin preparation inhibited a broad range of target bacteria belonging to both Gram-positive and Gram-negative groups, including *Micrococcus luteus*, *Listeria monocytogenes*, *Salmonella typhi, Staphylococcus aureus, Shigella flexneri, Pseudomonas fluorescens, P. aeruginosa, Vibrio* sp., and *Escherichia coli* O157:H7. Partially purified enterocin LD3 revealed a smear protein band with molecular weight between 3 and 7 kDa which showed a clear zone of inhibition after bioassay. Since strain LD3 has been isolated from a food source, it would be safe for human consumption in probiotic products or clinical applications.

Keywords Lactic acid bacteria · *Enterococcus hirae* LD3 · Probiotics · Bacteriocin · Food-borne pathogens

Introduction

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative rod or cocci and ferment carbohydrates to produce lactic acid during the growth. Different strains of LAB are considered to be essential for leavening of batter and also responsible for acid production in idli, dosa, and related products (Rhee et al. 2011; Gaaloul et al. 2015). Enterococci belong to LAB, and they are of importance in foods due to fermentation and their use as probiotics in humans and animals. According to the World Health Organization, probiotics are living microorganisms, which, upon ingestion, have beneficial effects to the host by improving the intestinal microflora. Several strains of LAB such as *Lactobacillus, Enterococcus*, and *Bifidobacterium* have been designated as probiotic bacteria (Rushdy and Gomaa 2013; Starke et al. 2014). Enterococci form an important part of food which is normally found in gastrointestinal tracts of both humans and animals. From the phylogenetic point of view, *Enterococcus hirae* is related to *Enterococcus faecium* (Lauková et al. 2008). Several strains of genus *Enterococcus* have been used as probiotics for the treatment of gastroenteritis in humans and animals (Bhardwaj et al. 2011; Hu et al. 2015).

The beneficial effects of probiotics include adherence to mucosal or epithelial cells of the host, acid and bile tolerance, safe, non-pathogenic, ability to survive in the gastrointestinal tract, improvement of intestinal microflora, reduction of cholesterol level, immune-stimulation, ability to

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synthesize and enhance the bioavailability of nutrients, and prevention of antibiotic-associated diarrhea (Franz et al. 2011; Turgis et al. 2013). Probiotic strains have the ability to survive in the harsh conditions of the gastrointestinal tract and adhere to the mucosal or epithelial cells which forms a defense against colonization by pathogenic microorganisms (Todorov et al. 2011; Yang et al. 2015). The safety of the bacteria used as probiotics must be assured, and data on the major strains in use so far indicate that they are safe. The use of enterococcal probiotics should, in view of the development of problematic lineages and the potential for gene transfer in the gastrointestinal tract of both humans and animals, be carefully monitored, and the advantages of using new strains should be considered in a well contemplated risk/benefit analysis (Franz et al. 2011).

LAB are also known to secrete bacteriocins, as part of their defense mechanism while growing in the environment. Bacteriocins are ribosomally synthesized peptides or proteins, and they inhibit the growth of closely related bacterial strains. However, few bacteriocins of LAB have also been reported to have a broader host range and inhibit Gram-negative bacteria (Tiwari and Srivastava 2008; Kumar et al. 2010; Gupta and Tiwari 2014b). LAB bacteriocins, i.e., nisin and pediocin PA-1/AcH, are used as food preservatives. The other bacteriocins also possess characteristics for application in human therapy, as potential supplements or replacements for currently used antibiotics (Rushdy and Gomaa 2013; Perez et al. 2014).

Franz et al. (2007) proposed a simplified classification scheme for enterocins: Class I lantibiotic enterocins, Class II small, non-lantibiotic peptides, Class III cyclic enterocins, and Class IV large proteins. Cytolysin belongs to Class I enterocins; it is a two-peptide bacteriocin and both structural subunits contain lanthionine residues. Class II can be subdivided into three subclasses: II.1, enterocin of the pediocin family (enterocin A, enterocin P, and bacteriocin 31); II.2, enterocins synthesized without a leader peptide (enterocin L50A/B, and enterocin Q); II.3, other linear, non-pediocin-type enterocins (enterocin B). Class III enterocins includes cyclic antibacterial peptides such as enterocin AS-48, and Class IV enterocins are large proteins such as enterolysin A.

To date, various strains of enterococci have been studied for their properties and probiotic applications (Hanchi et al. 2014). To the best of our knowledge, this is the first report on a probiotic strain of *Enterococcus hirae* isolated from dosa batter, a fermented food. Here, we describe the isolation, identification, and probiotic characterization of *E. hirae* LD3. Bacteriocin produced by strain LD3 was characterized for stability, host range, Tricine–SDS-PAGE, and bioassay.

Materials and methods

Bacterial strains, culture media, and growth conditions

All LAB strains were grown in MRS medium at 37 °C and maintained on TGYE agar medium. Reference LAB strains were obtained from Northern Regional Research Laboratory, ARS Culture Collection, USA. *Escherichia coli* (urogenic), *Pseudomonas fluorescens,* and *P. aeruginosa* were grown in Luria-Bertani broth at 37 °C. *Micrococcus luteus* MTCC 106, *Staphylococcus aureus, Salmonella typhi, Shigella flexneri, Listeria monocytogenes*, and *Vibrio* sp. were grown in nutrient broth at 37 °C, pH 7.0. *M. luteus* MTCC 106 was used as an indicator strain. The bacterial strains, their sources, and reason for their use in the present study are mentioned in Table 1. All the media components were purchased from Hi-Media (Mumbai, India), Sisco Research Laboratory (Mumbai, India), and Sigma-Aldrich (St. Louis, MO, USA).

Isolation, identification, and probiotic characterization of strain LD3

Strain LD3 was isolated from dosa batter and identified using biochemical and molecular techniques as previously described (Gupta and Tiwari 2014a). The probiotic properties were tested in terms of cell surface hydrophobicity, bile tolerance, bile salt hydrolase activity, survival in simulated conditions, and acid tolerance as described by Gupta and Tiwari 2014a.

Preparation and assay of crude bacteriocin

For preparation of a crude bacteriocin sample, strain LD3 was grown in MRS broth medium under static conditions at 37 °C for 18 h. After incubation, a cell-free supernatant was collected by centrifugation (10,000 *g*, 15 min, 4 °C) and filter-sterilized using a 0.2 μm membrane filter (mdi, Ambala, India). Culture supernatant was precipitated by ammonium sulphate (0–90 %) at 4 °C, solubilized in sodium acetate buffer (20 mM, pH 4.6), and dialyzed against the same buffer. This was used as the partially purified bacteriocin sample. The antimicrobial activity of bacteriocin was determined using the agar well diffusion assay (AWDA) method, performed by overlaying soft nutrient agar (0.8 %) inoculated with the indicator strain *M. luteus* (~10^6 CFU/ml) on the nutrient base agar plate. The wells cut out (6.0 mm diameter) on such plates were filled with 100 μl of bacteriocin. After overnight incubation, a halo was produced and diameter of zone of growth inhibition was measured. Antimicrobial activity was also determined in terms of AU/ml. One activity unit (AU) was defined as the reciprocal of the highest dilution of bacteriocin causing 50 % growth inhibition (Jimnez et al. 2013).

Table 1 Bacterial strains from different sources and reasons for their use in the present study

S. No.	Strain	Source	Reason for use
1.	*Micrococcus luteus* MTCC 106	IMTECH, India	Indicator strain
2.	*Lactobacillus curvatus* NRRL B-4562	ARS, USA	Target strain
3.	*L. delbrueckii* NRRL B-4525	ARS, USA	Target strain
4.	*L. acidophilus* NRRL B-4495	ARS, USA	Target strain
5.	*L. plantarum* NRRL B-4496	ARS, USA	Target strain
6.	*Lactococcus lactis* subsp. *lactis* NRRL B-1821	ARS, USA	Target strain
7.	*Lactococcus lactis* subsp. *cremoris* NRRL B-634	ARS, USA	Target strain
8.	*Enterococcus faecium* NRRL B-2354	ARS, USA	Target strain
9.	*Enterobacter cloacae* NRRL B-14298	ARS, USA	Target strain
10.	*Staphylococcus aureus*	Department of Microbiology, Delhi University	Pathogen
11.	*Escherichia coli* O157:H7	Rutgers State University	Pathogen
12.	*Pseudomonas fluorescens*	Department of Genetics, Delhi University	Pathogen
13.	*P. aeruginosa*	Department of Microbiology, Delhi University	Pathogen
14.	*Salmonella typhi*	Department of Microbiology, Delhi University	Pathogen
15.	*Shigella flexneri*	Department of Microbiology, Delhi University	Pathogen
16.	*Listeria monocytogenes*	Department of Microbiology, Delhi University	Pathogen
17.	*Vibrio* sp.	Department of Microbiology, Delhi University	Pathogen

Characterization of bacteriocin

Effect of pH and heat treatment

To test the effect of pH and heat, the bacteriocin sample was resuspended in a 1:1 ratio of different buffer solutions ranging from pH 2.0–10.0 (HCl-KCl, 50 mM, pH 2.0 and 4.0; phosphate buffer, 50 mM, pH 6.0 and 7.0; Tris-Cl, 50 mM, pH 8.0 and 10.0) and incubated for 2 h at 37 °C. The residual activity was monitored by AWDA. In the control set, only buffers (of each pH) were used in place of bacteriocin. For thermo-stability, the sample was treated to 80 and 100 °C for 15 min in a water bath, and at autoclaving temperature (121 °C for 15 min) prior to measure activity. The residual activity of the treated samples was compared with that of control grown at 37 °C.

Effect of organic solvents, surfactants and detergents

In a separate experiment, ethanol, methanol, isopropanol, acetone, ethyl acetate, dodecyl sulphate (SDS), Tween 80, urea, Triton X-100 were mixed at a final concentration of 1 % (v/v or w/v as appropriate) with the crude bacteriocin preparation. In sterile distilled water, the untreated cell-free supernatant served as control. All samples were incubated at 37 °C for 2 h and tested for antimicrobial activity by AWDA.

Effect of hydrolytic enzymes on bacteriocin activity

To determine the chemical nature of bacteriocin, the sample was treated with different enzymes at a final concentration of 1.0 mg/ml. These were amylase, lipase, trypsin, pepsin, papain, and proteinase K (Sigma-Aldrich, USA). Following incubation at 37 °C for 2 h, enzyme activity was terminated by heating the sample at 100 °C for 5 min. The untreated sample was used as control and the residual activity was measured by AWDA.

Mode of action and host range

About 1.0×10^6 cells of active growing culture of indicator strain *M. luteus* were resuspended in fresh nutrient broth medium containing approximately 10 AU/ml of crude bacteriocin preparation and incubated at 37 °C. Samples were removed at regular time intervals of 2 h up to 8 h for the determination of viable cell counts (CFU/ml). Control was grown without bacteriocin. To determine the effect of the antimicrobial substances on related as well as other bacteria, the culture supernatant was tested against different LAB, Gram-positive, and Gram-negative bacteria by AWDA. The tested strains were grown in their respective media.

Tricine SDS-PAGE and bioassay

To determine the molecular mass, the partially purified bacteriocin preparation was run on Tricine SDS-PAGE using the protocol as described by Schägger and von Jagow (1987) with some modifications. These included an additional 20 % layer of separating gel. Using a vertical gel apparatus with 4 % stacking gel, 10 and 13 % spacer, and 16.5 and 20 % separating gel, bacteriocin sample along with molecular weight marker (Bangalore Genei, India) was run at 100 V for 5 h.

After electrophoresis, the gel was divided into two halves. One half containing molecular weight marker was stained with CBB R-250 and the other was overlaid with the indicator strain (~10^6 CFU/ml) in TGYE soft agar to detect inhibitory activity against indicator strain (Tiwari and Srivastava 2008).

Results

Isolation and identification

Strain LD3 was isolated from dosa batter and identified using morphological, biochemical, and molecular techniques. The strain LD3 was Gram-positive, catalase negative, and coccus-shaped. It was also able to produce ammonia from arginine and ferment different carbohydrates such as galactose, raffinose, trehalose, melibiose, fructose, dextrose, maltose, cellobiose, sodium gluconate, salicin, inulin, xylitol, mannose, sucrose, and esculin. Based on these tests, strain LD3 was found to belong to genus *Enterococcus* (Holt et al. 1994). Species level identification was done using 16S rDNA amplification and sequencing. The size of the amplified PCR products was found to be approximately 0.9 kb (Fig. 1a). The PCR products were sequenced at a DNA sequencing facility available at University of Delhi South Campus, New Delhi. Out of 0.9 kb fragment amplified, 568 bp sequence was obtained and used for homology search for 16S rDNA sequences available in database using BLAST, NCBI. Phylogenetic analysis of ten sequences closer to the sequence obtained based on the BLAST result was found to have 99 % similarity with *Enteorococcus hirae* ATCC 9790 as shown in Fig. 1b. The sequence was submitted to GenBank, NCBI with accession number KC710968.

Probiotic properties

Cells of strain LD3 showed the positive response in spontaneous and auto-aggregation assay. Cell-to-cell adherence was monitored in the microscopic examination as shown in Fig. 2a and b. Such adherence was observed more in the presence of ammonium sulphate as compared to without salt. The aggregation of cells was observed even at the lowest concentration (0.007 M) of salt used (SAT value 0.007 M) (Fig. 2c). Cell adherence was also observed on glass surface which indicated the strong ability of strain for cell surface hydrophobicity. The cell adherence of strain LD3 was also quantified using MATH test and found to be 30 % which also confirmed its hydrophobic potential. Therefore, strain LD3 was able to demonstrate cell surface hydrophobicity which is a good sign of probiotic property.

Strain LD3 showed 84 % survival after treatment of cells at pH 2.0 in the presence of pepsin which suggested the ability to survive at low pH in a gastric environment. The ΔpH value (0.4 U) of the strain was achieved during 3–5 h and, therefore, strain LD3 could be classified as a medium acidifying strain. Strain LD3 showed tolerance to pH 2.0, though there was a little decrease in viability count. Nowadays use of antacids is very common, thus it was important to observe the effect of antacid on the survival of probiotic strains. Strain LD3 was able to survive 100 % in the presence of ENO (GlaxoSmithKline, India), and only marginal (1 %) loss in viability was recorded in the presence of Ranitidine hydrochloride (J. B. Chemicals & Pharmaceuticals, India). This suggested the survival of cells under simulated conditions. In the highly competitive environment of the human intestinal tract, bile salt hydrolase (BSH) activity is a selective advantage on probiotic strains. Growth of strain LD3 in the presence of taurodeoxycholic acid, $CaCl_2$, and precipitation zone confirmed positive BSH activity. *Lactobacillus plantarum* LR/14 and *Enterococcus faecium* LR/6 were used as a positive and

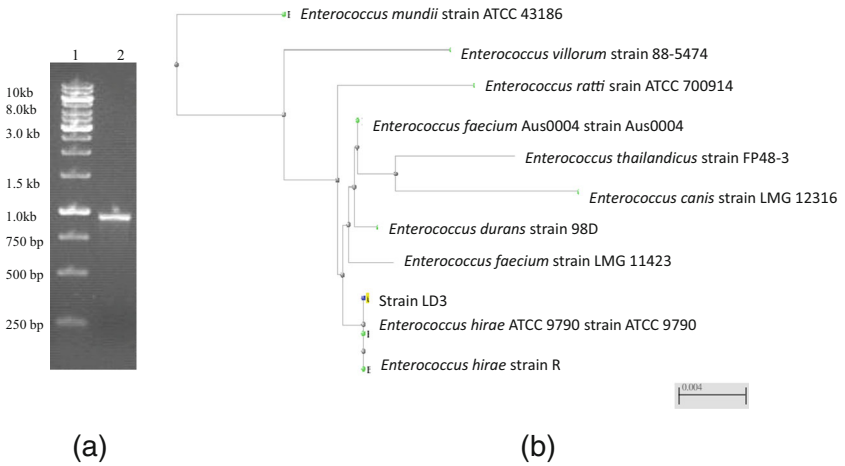

Fig. 1 **a** Agarose gel electrophoresis showing DNA molecular weight marker (lane 1) and 16S rDNA fragment amplified by PCR from genomic DNA of strain LD3 (lane 2). **b**. Phylogenetic tree based on 16S rDNA sequence amplified from genomic DNA of strain LD3 and other strains as obtained from BLAST result

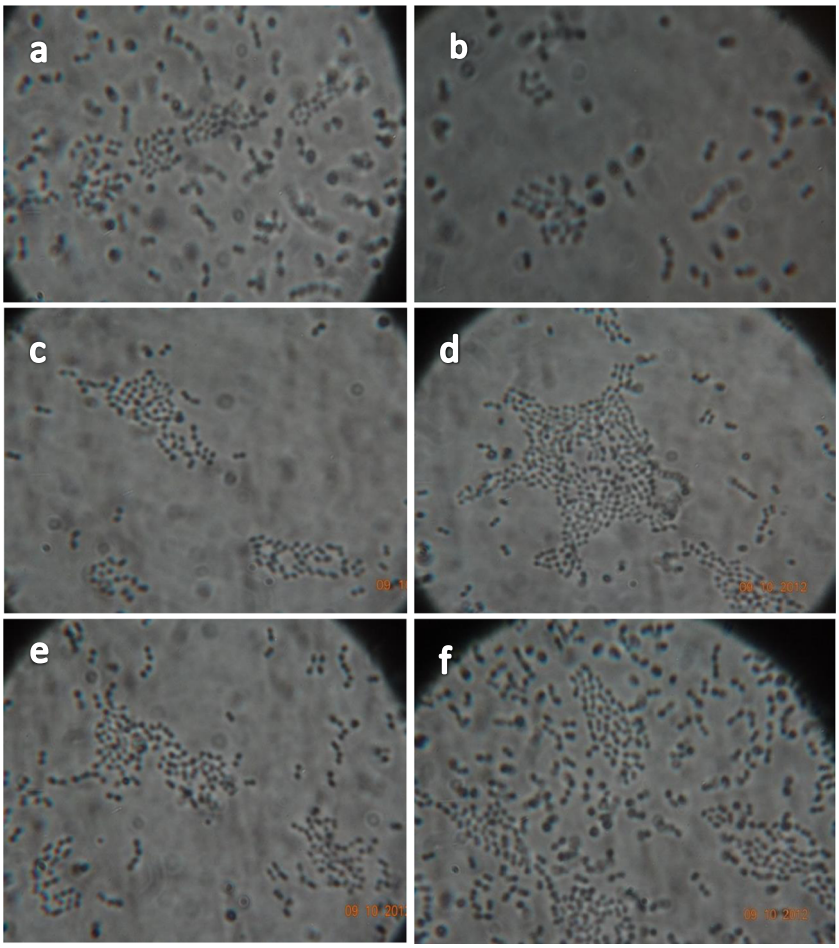

Fig. 2 Demonstration of cell surface hydrophobicity by spontaneous aggregation (**a**), auto-aggregation (**b**), and salt aggregation tests in 0.007 (**c**), 0.5 (**d**), 1.0 (**e**), and 2.0 M (**f**)

negative control, respectively. The growth of strain LD3 in the presence of conjugated bile acid (taurocholic acid) and mixture of conjugated and deconjugated bile acid (oxgall) is an important criterion for assessing its survival in the intestine. Strain LD3 was found to be resistant to kanamycin and gentamycin, but sensitive to tetracycline, erythromycin, and chloramphenicol suggesting efficient probiotic applications.

Growth kinetics and production of bacteriocin

Growth response, level of bacteriocin production, and change in the pH were monitored at different stages of growth of strain LD3. It was found to be a fast growing strain which reached log phase at 3 h and completed at the end of 5 h, and thereafter entered into the stationary phase. The fast growth of strain LD3 would be an advantage during the recovery of bacteriocin, production of which started at 3 h and reached a maximum at 6 h (Fig. 3). For bacteriocin production, culture-free supernatant was collected at every step and antimicrobial activity was determined in terms of size of growth inhibition zone (mm) using AWDA. A decreasing trend in the pH was found while the growth proceeded and dropped to pH ~4.5 from the initial pH 6.8 of the culture medium.

Characterization of bacteriocin

Crude bacteriocin preparations of strain LD3 was active in the range of pH 2.0 to 6.0. The antibacterial activity of bacteriocin was 100 % at pH 2.0 and 4.0, 92 % at pH 6.0, but there was complete loss in activity between pH 7.0 and 10.0 (Table 2).

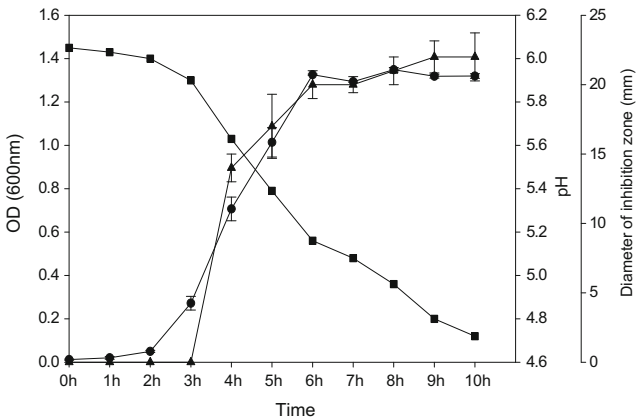

Fig. 3 Growth response of *E. hirae* LD3 in the MRS medium (●), change in pH (■), and antimicrobial activity (▲) against indicator strain *M. luteus*

Table 2 Characterization of crude bacteriocin preparation and probiotic properties of *Enterococcus hirae* LD3

S. No.	Treatment	Bacteriocin activity (mm)
1.	pH 2.0	26±0.57
	pH 4.0 (control)	26±0.45
	pH 6.0	24±0.41
	pH 7.0	0.0
	pH 8.0	0.0
	pH 10.0	0.0
2.	Control	26±0.28
	80, 100, and 121 °C for 15 min	26±0.15
3.	Control	26±0.28
	Ethanol, methanol, isopropanol, acetone, ethyl acetate, SDS, Tween-80, urea, Triton X-100	26±0.45
4.	Control	26±0.28
	Catalase, amylase, and lipase	26±0.57
	Pepsin	20±0.45
	Trypsin	18±0.15
	Proteinase K	14±0.28
	Papain	16±0.24
5.	*Micrococcus luteus* MTCC 106	22±0.28
	Lactobacillus curvatus NRRL B-4562	11±0.45
	L. delbrueckii NRRL B-4525	12±0.57
	L. acidophilus NRRL B-4495	18±0.28
	L. plantarum NRRL B-4496	0.0
	Lactococcus lactis subsp. *lactis* NRRL B-1821	0.0
	Lactococcus lactis subsp. *cremoris* NRRL B-634	20±0.28
	Enterococcus faecium NRRL B-2354	19±0.28
	Enterobacter cloacae NRRL B-14298	11±0.06
	Staphylococcus aureus	13±0.03
	E. coli O157:H7	18±0.06
	Pseudomonas fluorescens	17±0.15
	P. aeruginosa	13±0.28
	Salmonella typhi	14±0.15
	Shigella flexneri	12±0.06
	Listeria monocytogens	13±0.06
	Vibrio sp.	12±0.23

Buffers of related pH were used as control and did not show any growth inhibition. Incubation of crude preparation at different temperatures also led to no loss in activity as 100 % activity was recorded even after boiling and autoclaving. Thus, the antimicrobial compound was found to be highly thermo-stable. Similar stability was observed when the sample was treated with different organic solvents, surfactants, and detergents. The bacteriocin activity was not affected by the reagents used. Activity remains the same after treatment with that of untreated bacteriocin (Table 2). The antimicrobial activity of the crude bacteriocin preparation was retained after catalase treatment suggested antibacterial activity was not due to H_2O_2. When the bacteriocin sample was treated with proteolytic enzymes such as pepsin, trypsin, proteinase K, and papain, the activity of the inhibitory compound was reduced, but amylase and lipase did not influence the activity of bacteriocin LD3 (Table 2). This suggested the proteinaceous nature of the inhibitory compound.

To monitor the effect of crude bacteriocin preparation on active cells of *M. luteus*, inhibition of growth was measured in terms of cfu/ml. The addition of crude bacteriocin preparation (10 AU/ml) to ~1×10^6 cells of the indicator strain caused a significant decrease in cellular viability. Killing of the cells started within 1 h after the addition, and by 8 h a more than 40 % loss in viable count was recorded as compared with control (Fig. 4). This suggested the bactericidal mode of action of the crude preparation of bacteriocin LD3. It inhibited both Gram-positive and Gram-negative bacteria as mentioned in Table 2. The result was found to be more encouraging when the crude bacteriocin preparation was also able to inhibit some

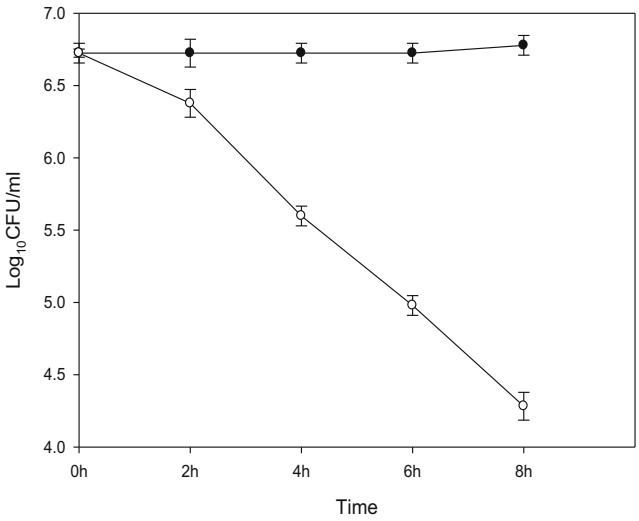

Fig. 4 Effect of bacteriocin on the viability of *M. luteus* cells without (●) and after treatment (○)

human and food-borne pathogens such as *Staphylococcus aureus, Pseudomonas fluorescens, P. aeruginosa*, *Salmonella typhi*, *Shigella flexneri, Listeria monocytogenes, E. coli* O157:H7, and *Vibrio* sp. (Table 2).

To determine the molecular mass, concentrated bacteriocin preparation was loaded on Tricine–SDS-PAGE. Though protein was not resolved into a sharp band, it showed a smear between 3.48 and 7.68 kDa. The inhibition zone corresponding to the protein band was observed when the replicate of the gel was applied for the bioassay as shown in Fig. 5. This further confirmed the proteinaceous nature and antimicrobial activity present in the crude preparation of bacteriocin LD3.

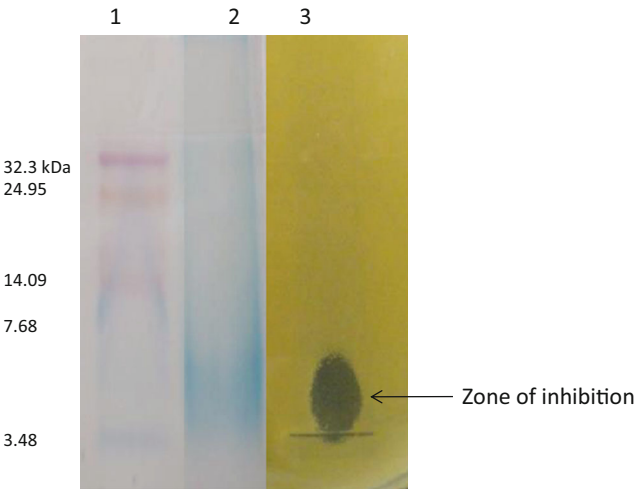

Fig. 5 Tricine–SDS-PAGE of partially purified bacteriocin sample of strain LD3. Lane 1: Molecular weight marker, Lane 2: partially purified bacteriocin, Lane 3: Zone of growth inhibition due to antimicrobial activity corresponding to the position of protein band in lane 2. The gel was overlaid with viable cells of *M. luteus* (~10^6 CFU/ml) in TGYE soft agar. Incubation was done at 37 °C for 18 h

Discussion

During the few years, there has been an increase in food-borne illness caused by microbial contamination. This led to the discovery of inhibitory substances active against food-borne pathogens. Therefore, the main goal of this study was the isolation and probiotic characterization of bacteriocin-producing LAB from food items. Dairy and other fermented food products are major sources of lactic acid bacteria (Rhee et al. 2011). Keeping this in mind, in our previous study, 26 strains of lactic acid bacteria were isolated from different fermented food products such as cheese, raw milk, Jalebi batter, Jalebi, dosa batter, Uttpam, pickles, and Bhature (Gupta and Tiwari 2014a). Among these, strain LD3 isolated from Dosa batter, a fermented food, was found to demonstrate higher antimicrobial activity and, therefore, was selected for further study.

Production of ammonia from arginine is an important feature of lactic acid bacteria and was also demonstrated by strain LD3. It was able to ferment different carbohydrates and the fermentation pattern thus obtained was compared with reference strains present in Bergey's Manual (Holt et al. 1994). Based on these tests, strain LD3 was found to belong to genus *Enterococcus*. Species level identification was done using 16S rDNA amplification and sequencing. Phylogenetic analysis of ten sequences closer to the sequence obtained based on the BLAST result was found to have 99 % similarity with *Enteorococcus hirae* ATCC 9790. The isolation and identification of bacteriocin-producing LAB from raw cow milk has also been demonstrated by Gaaloul et al. 2015. Different strains of *Lactobacillus*, isolated from traditional sorghum-based fermented foods, have demonstrated potential probiotic properties (Poornachandra Rao et al. 2015).

To meet the basic criteria of a probiotics, hydrophobicity and adherence of strain LD3 was examined. Cells of strain LD3 showed a positive response in spontaneous and auto-aggregation assays. Such adherence was observed more in the presence of ammonium sulphate. Cell adherence on a glass surface also indicated the strong ability of the strain for cell surface hydrophobicity, which is a probiotic property. Generally, enterococci have low hydrophobicity for n-hexadecane, as 92.9 % of *Enterococcus faecium* and 79.2 % of *Enterococcus faecalis* strains showed low hydrophobicity (0–30 %) (Bhardwaj et al. 2011). Cell surface hydrophobicity is a non-specific interaction between microbial cells and host involving cell surface proteins and lipoteichoic acids (Todorov et al. 2011). *Lactobacillus rhamnosus* GG showed only 32 % adherence. From numerous ecological niches, mainly in the human gut, bacterial aggregation of the same strains (auto-aggregation) is considered to be more important because of the presence of probiotics. This is an important factor by which probiotic strains determine the ability to adhere to the oral cavity, gastrointestinal, and urogenital tract (Divya et al.

2012). The strain under investigation was able to demonstrate cell surface hydrophobicity, which is a good sign of probiotic property.

For probiotic applications, a strain needs to resist in the gastrointestinal environment, and therefore, strain LD3 was tested in different simulated conditions. Strain LD3 showed survival and tolerance after treatment of cells at pH 2.0 in the presence of pepsin, which suggested the ability to survive at low pH in a gastric environment. Upon oral administration, a major physiological challenge that probiotic strains face is the pH of gastric juice (pH 2.0–3.0), which normally destroys most ingested microorganisms. Thus, for probiotic strains, tolerance to acidic conditions is an important selection criterion. In the case of enterococci, reports for in vitro tolerance are scarce (Bhardwaj et al. 2011). Gardiner et al. (1999) has shown that survival of probiotic strain *E. faecium* Fargo 688 in porcine gastric juice at pH 2.0 was only 8 min, whereas good survival was found in *E. faecium* strains after exposure at pH 2.5 (Ruiz-Moyano et al. 2008).

Nowadays, use of antacids is very common. Thus, it is important to observe the effect of antacid on the viability of probiotic strains. Survival of strain LD3 in simulated conditions could provide an advantage to pass through gastrointestinal tract (GIT) if applied in probiotic products. Studies about the effect of antacids on probiotic strains are limited and should be carried out to understand the potential applications of probiotic strains (Ghosh et al. 2008; Gupta and Tiwari 2014a). In the highly competitive environment of the human intestinal tract, bile salt hydrolase (BSH) activity is a selective advantage on probiotic strains which is demonstrated by strain LD3. Wijaya (2002) stated that 67.4 % of *E. faecalis*, 58.1 % *E. faecium,* and 50 % *E. durans* strains exhibited BSH activity against taurodeoxycholic acid.

The growth of strain LD3 in the presence of conjugated bile acid (taurocholic acid) and mixture of conjugated and deconjugated bile acid (oxgall) is an important criterion for assessing its survival in intestines. Besides the resistance against strong acidic conditions of the stomach, probiotic strains have to resist bile salts in the GIT. Therefore, bile tolerance is an important property required for survival of probiotic strains (Walker and Gilliland 1993; Bhattacharya and Das 2010). Bile salts are surface-active chemicals synthesized in the liver from the catabolism of cholesterol entering the duodenal section of small intestine (Rushdy and Gomaa 2013). Enterococci are well identified to be commensals of the GIT of human and animals, and they show an interaction with bile salts. Thus, it is not surprising that *Enterococcus* spp. are resistant to bile salts (Bhardwaj et al. 2011). Several reports were given about the strongly resistant condition of *E. faecium* to bile salts (Harun-ur-Rashid et al. 2007; Ruiz-Moyano et al. 2008), indicating the potentiality to survive in the GIT environment.

Lactobacilli were generally susceptible to those antibiotics which inhibit protein synthesis, such as tetracycline, erythromycin, chloramphenicol, and more resistant to aminoglycosides (kanamycin and gentamycin) and glycopeptides. *E. faecium* and *E. faecalis* isolates were highly resistant to aminoglycosides (kanamycin and gentamycin) (Birri et al. 2013). The absence of antibiotic resistant genes in bacteria is a positive trait for probiotics (Rushdy and Gomaa 2013; Poornachandra Rao et al. 2015). For probiotic strains, it must be that they should not carry transferable antibiotic resistance genes (Marroki et al. 2011).

Growth response, level of bacteriocin production and change in the pH were monitored at different stages of growth of strain LD3. Fast growth of strain LD3 would be an advantage during the recovery of bacteriocin. A decreasing trend in the pH suggested the acid production throughout the growth. The crude bacteriocin preparation of strain LD3 was active in an acidic pH range and showed thermo-stability even after boiling and autoclaving. The activity of bacteriocins JJ18 and JJ60 remained stable from pH 2 to 6, whereas at alkaline pH of 8 to 12, a loss of activity by 25–50 % was observed (Agaliya and Jeevaratham 2013) while enterocin A5-11 was active in acid to alkaline conditions at pH 2.0 to 10.0 (Batdorj et al. 2006). The thermo-stability of bacteriocin offers promising to sterilize the food products during processing and could be stored at room temperature (Sankar et al. 2012). The activity of bacteriocin FPTLB3 remained constant after heating at 121 °C for 60 min, but later declined (Banerjee et al. 2013). Bacteriocins JJ18 and JJ60 were 100 % resistant to temperatures ranging from 4 to 60 °C, but there was loss of 50 % activity from 80 to 100 °C after 2 h and at 121 °C for 15 min the bacteriocin exhibited only 50 % activity (Agaliya and Jeevaratham 2013). Similar stability was observed when the crude bacteriocin preparation was treated with different organic solvents, surfactants, and detergents. Bacteriocins JJ18 and JJ60 were stable when treated with Tween 80, Triton X-100, and SDS at 1 % (w/v) or EDTA. Similar results were recorded for plantaricin C19, LD1, and bacteriocin AMA-K (Atrih et al. 2001; Todorov et al. 2007; Gupta and Tiwari 2014b).

The antimicrobial activity of the crude bacteriocin preparation was retained after catalase treatment which negated the involvement of H_2O_2 for activity. Similar results were also reported by Todorov and Dicks (2004) and Hernandez et al. (2005). When the crude bacteriocin preparation of strain LD3 was treated with different proteolytic enzymes, activity of the inhibitory compound was reduced, but amylase and lipase did not influence the activity, suggesting the proteinaceous nature of inhibitory compound. Probably there is no role of carbohydrate or lipid moieties for the activity of bacteriocins. Such loss in activity has also been shown by other bacteriocins, e.g., bacteriocin LCL01 activity was lost when treated with proteinase K and trypsin (Saidi et al. 2011). The appearance of smear protein band on tricine SDS-PAGE between 3.48 and 7.68 kDa suggested small protein and activity was

demonstrated by bioassay. The molecular weight of enterocins produced by *E. faecium* GGN7 was in the range of 3.2 to 5.4 kDa, which was analyzed by MALDI-TOF/MS, but Tricine–SDS-PAGE did reveal protein bands (Gaaloul et al. 2015). The purification of bacteriocin LD3 to homogeneity, mass analysis, and amino acid sequencing have been further planned for molecular characterization.

A significant decrease in cellular viability of the indicator strain suggested the bactericidal mode of action of the crude bacteriocin preparation of strain LD3. A bactericidal effect was also reported in bacteriocin AMA-K (Todorov et al. 2007) and LCL01 (Saidi et al. 2011), whereas bacteriocin from *L. curvatus* A61 was found to be bacteriostatic (Ahmadova et al. 2013). Bacteriocin preparation from strain LD3 inhibited both Gram-positive and Gram-negative bacteria. The result was more encouraging when it was found to inhibit some human and food-borne pathogens. It would be an additional benefit for the application of strain LD3 in probiotic food products.

Conclusions

Enterococcus hirae LD3 isolated from dosa batter, was identified using biochemical and molecular methods. The growth response of strain LD3 was typical sigmoidal where growth associated bacteriocin production was recorded. Crude bacteriocin preparation was stable in acidic pH, high temperatures, and in the presence of various organic solvents, surfactants, and detergents tested. Antimicrobial activity was reduced in the presence of proteolytic enzymes, but not affected by amylase and lipase, suggesting the proteinaceous nature of the inhibitory compound. It showed bactericidal mode of action against indicator strain *M. luteus*. Antimicrobial activity was recorded for certain food-borne as well as pathogenic bacteria, suggesting its application in pharmaceutical industries. Such properties of the crude bacteriocin preparation prompted us to purify to homogeneity and characterize for various molecular properties and mode of action in our laboratory. The characteristics of the strain LD3 such as cell surface hydrophobicity, acid tolerance, bile tolerance, BSH activity, survival in simulated condition with antacid, and inhibition of pathogenic bacteria, may be used for the development of a probiotic strain for industrial applications.

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Conflict of interest All authors declare no conflict of interest.

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